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Three Novel Single Nucleotide Polymorphisms of the 3-hydroxy-3-methylglutaryl Coenzyme A Reductase Gene Associated with Egg-production in Chicken*

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Different single-strand conformation polymorphism (SSCP) patterns of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGCR*) gene amplified by six pairs of primers were sequenced. Comparisons among the six nucleotide sequences of three genotypes revealed three base substitutions (G-789-A, C-937-G, and A-2316-C) in the *HMGCR* gene. The genotypes of single nucleotide polymorphism 1 (SNP1) and SNP3 were significantly associated with the number of eggs ($P<0.01$), and the different genotypes of SNP2 did not significantly influence the number of eggs. The genotypes of all three SNPs were not related to the hatching rate of fertilized eggs ($P>0.05$). Of the three SNPs of the *HMGCR* gene, those genotypes with lower plasma very low-density lipoprotein (VLDL) concentrations had higher egg production. The 300-day egg production of the six studied haplotypes showed three levels ($P<0.01$). The ADE and ACE haplotypes showed lower egg production than the other haplotypes. The ADE haplotype had the highest plasma VLDL concentration, and the ACE haplotype had the lowest plasma VLDL concentration. We concluded that specific variants of the *HMGCR* gene are associated with chicken egg production, and haplotypes with higher and lower plasma VLDL concentrations showed lower egg production than other haplotypes.

Key words: 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR); single nucleotide polymorphism (SNP); egg production; chicken.

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3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) is a key enzyme in cholesterol homeostasis and catalyzes the rate-limiting step in cholesterol biosynthesis (GOLDSTEIN & BROWN 1990). Several polymorphisms have been identified in the *HMGCR* gene locus, and two tightly linked single nucleotide polymorphisms (SNPs) were found to be significantly associated with a difference in the change of the serum lipid response to pravastatin treatment (CHASMAN *et al*. 2004). HMGCR inhibition by statins has been shown to downregulate angiotensin II type 1 receptor expression, and the effect is reversed by mevalonate and by geranylgeranyl-pyrophosphate, which are products of HMGCR activity (STREHLOW *et al*. 2000; ICHIKI *et al*. 2001; PARK *et al*. 2002). A significant correlation has been shown between a SNP in intron 5 of the *HMGCR* gene and carcass traits, down characteristics, and meat quality traits by polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) in geese (ZHONG *et al*. 2008). Two SNPs in exon 17 and exon 18 of the chicken *HMGCR* gene, identified by PCR restriction fragment length polymorphism, were associated with growth, carcass traits, meat quality, and lipid concentration (WEI *et al*. 2012).

Recently, a common SNP in intron 13 of *HMGCR* (rs3846662) has been reported to be a functional variant that influences the alternative splicing of exon 13 (BURKHARDT *et al*. 2008). In that study, lymphoblastoid cells from subjects that were homozygous for the major A allele showed higher

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levels of an alternatively spliced isoform missing exon 13 than those from homozygotes for the minor G allele. The alternatively spliced isoform of *HMGCR* has been detected in various human tissues, including kidney, liver, heart, spleen, lung, placenta, skeletal muscle, ovary, peripheral blood leukocytes, small intestine, bone marrow, brain, spinal cord, testes, thyroid gland, and uterus (JOHNSON *et al*. 2003; BURKHARDT *et al*. 2008). The proportion of the alternative splicing variant to the total *HMGCR* mRNA has been suggested to be tissue-specific (BURKHARDT *et al*. 2008). In a recent pharmacogenetic study, the *in vitro* upregulation of alternative splicing induced by statin treatment was inversely associated with the *in vivo* statin response and was partly determined by the genotypes of rs3846662 (MEDINA *et al*. 2008).

So far, most studies have focused on the association between polymorphisms of the human *HMGCR* gene and cardiovascular disease. Studies have shown that the *HMGCR* gene directly regulates serum lipoprotein metabolism through a feedback mechanism (GOLDSTEIN & BROWN 1990). Although genetic variation is known to affect blood lipids (HUBACEK *et al*. 1999; PLAT & MENSINK 2002; TONG *et al*. 2004), no study has examined the effect of *HMGCR* polymorphisms on reproductive traits. The purpose of this study was to investigate SNPs in the *HMGCR* gene and their association with chicken reproductive traits.

Material and Methods

Animals and trait determination

A total of 400 female chickens were randomly selected in this study from the F5 generation of Qing-Jiao-Ma breeding chickens. All chickens were fed *ad libitum* and managed under the same conditions. Venous blood samples (1 ml) were taken, and ethylenediaminetetraacetic acid was added as an anticoagulant. Then, the blood samples were kept at -20°C until they were used for DNA extraction, and the supernatant plasma was kept at -80°C to measure the very low-density lipoprotein (VLDL) concentration. Egg production at 300 days and the hatching rate of fertilized eggs from 55 to 60 weeks were determined.

Measurement of plasma VLDL concentrations

The VLDL concentration in the supernatant plasma was measured using a chicken VLDL enzyme-linked immunosorbent assay kit (Huijia, China). As described in the instructions, a microtiter plate was pre-coated with an antibody specific to VLDL. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of VLDL in the samples was determined by comparing the optical density of the samples to that of the standard curve.

DNA extraction and primer design

Genomic DNA was extracted from blood of the chickens by the phenol-chloroform method. The primers were designed using Oligo software according to the *HMGCR* gene sequence of chicken in GenBank (GenBank accession No. NC_006127.2) and are shown in Table 1. All primers were provided by Sangon Biotech Co. Ltd. (Shanghai, China).

Table 1

Primer sequences and corresponding PCR product size

Primer number	Primer sequences (5'→3')	Annealing temperature	Product size (bp)	Location (bp)
P1	F:TTTCAGAACCTAAGGCAACT R:AACCACACCACTAAAACCAG	59	328	1057-1384
P2	F:TGAAGAGAGGATGAAGCGA R:TGTATGGAACACAAAGGGG	58	521	463-983
P3	F:TCTGTGCTGTGTGGATGA R:CGAGTTGCCTTAGGTTCT	57	516	563-1078
P4	F:ATGTAGATTAGGGGTCAGGAAA R:AGAGGAAAGAAATGGTGTTAGG	60	522	5118-5639
P5	F:CTGTATTCTGCGTGGTGTTA R:CATCTTGAGGTCATCTGGTC	57	422	2187-2608
P6	F:CGATTCTTGTAGGCTGTTTT R:TCATTTTGACCCTCTGTGTT	56	506	4326-4831

PCR amplification and PCR-SSCP

PCR amplifications were performed using the following protocol for 35 cycles: denaturation for 30 s at 95°C, annealing for 30 s at 54 to 64°C, and extension for 30 s at 72°C; this was followed by a final extension for 5 min at 72°C. PCR products were visualized by 1% agarose electrophoresis.

PCR products were denatured at 98°C for 8 min and placed on ice. Electrophoresis was performed on 8 to 15% nondenaturing polyacrylamide gels. After electrophoresis, gels were stained with silver (Refined Chemical Plant, Shanghai, China). Some of the samples (15%) were replicated to detect the mutations of every amplified PCR fragment by PCR-SSCP analysis, and the concordance rate ranged from 99.1 to 100% for various amplified PCR fragments.

PCR products showing abnormal mobility under SSCP analysis were sequenced by Sangon Biotech Co. Ltd. Sequencing results were analyzed for gene mutations with Chromas 2.22 software (Technelysium Pty. Ltd., QLD, Australia).

Statistical analysis

The genotype and allele frequencies were computed according to electrophoresis results. The genetic variation of the *HMGCR* gene was calculated using PopGene 32 software (population genetic analysis, version 1.31). The polymorphic information content (PIC) and heterozygosity (He) were calculated according to established formulas (NEI 1978; BOTSTEIN *et al*. 1980). The association analysis of polymorphisms with traits was carried out using the GLM model of SAS 9.1 (SAS Institute, Cary, NC, USA). Comparative analysis between different genotypes was performed using the least squares method. The results are listed as means ± standard error.

Results

Polymorphism detection by PCR-SSCP

Fragments of the chicken *HMGCR* gene that were 328, 521, 516, 522, 422, and 506 bp were amplified by PCR using six pairs of primers. The genetic polymorphism of the population was detected by SSCP (Figs 1-3). Three single mutation sites were identified in those fragments at the site of 789 bp (G-789-A) in intron 1(SNP1), 937 bp (C-937-G) in intron 1 (SNP2), and 2316 bp (A-2316-C) in intron 4 (SNP3).

Genotypic and allelic frequencies

Three genotypes were detected in three DNA fragments of the *HMGCR* gene. Two SSCP patterns were obtained for each genotype. Because *HMGCR* is a sex-linked gene and the chickens in this study are all female, the genotypic and allelic frequencies are the same. The genotypic and allelic frequencies and the PIC of the locus are shown in Table 2. Allele C was predominant in the population. The maximum value of the C_ genotypic frequency and the minimum value of the D_ genotypic frequency were obtained for the *HMGCR* fragment that was amplified by P3. The distribution of alleles E and F was balanced. The values of the E_ and F_ genotypic frequencies were close. Analysis of Hardy-Weinberg equilibrium indicated disequilibrium ($P<0.05$).The three sites showed moderate polymorphism ($0.25<PIC<0.5$).

Association between the variants of the *HMGCR* gene and reproduction traits in chickens

As shown in Table 3, the SNP1 genotypes were significantly associated with the number of eggs ($P<0.01$), and the number of eggs was significantly higher in chickens with the B_ genotype than the C_ genotype. In contrast, the SNP1 genotypes were not significantly associated with the hatching rate of fertilized eggs ($P<0.05$). The SNP3 genotypes had a significant impact on the number of eggs ($P<0.01$). Multiple comparisons revealed that the E_ genotype was associated with significantly higher egg production than the F_ genotype. Different SNP2 genotypes did not significantly influence the number of eggs ($P>0.05$). The genotypes of the three SNPs were not significantly associated with the hatching rate of fertilized eggs ($P>0.05$). Additionally, of the three SNPs of the *HMGCR* gene, the genotypes with lower plasma VLDL concentrations had higher egg production (Table 3).

Eight haplotypes were obtained using the three SNPs. Two haplotypes, BDE and BDF, were removed from the analysis because they did not meet the statistical requirements (Table 4). The 300-day egg production of the 6 haplotypes showed 3 levels ($P<0.01$). The BCE haplotype had the highest number of eggs (106.11); the ACF, ADE, ADF, and BCF haplotypes showed a moderate number of eggs (93.19, 92.12, 95.33, and 95.27, respectively), and the ACE haplotype had the lowest number of eggs (84.90). Hatching rates of fertilized eggs of the six haplotypes did not differ ($P>0.05$).

Of the six haplotypes, the ADE haplotype had the highest plasma VLDL concentration, and the ACE haplotype had the lowest plasma VLDL concentration (Table 4). Additionally, these two haplotypes showed lower egg production than other haplotypes.

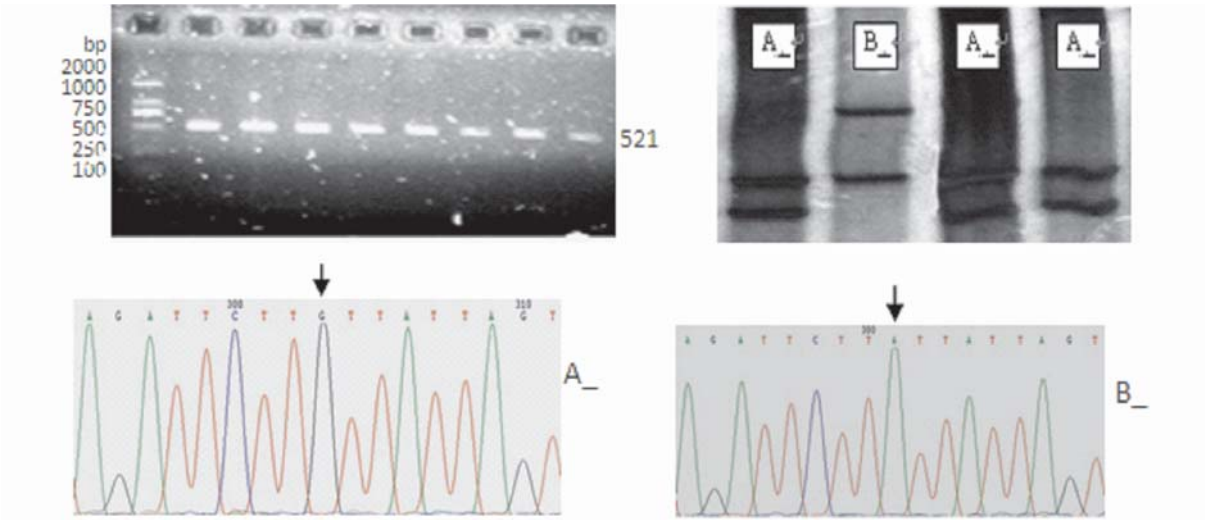

Fig. 1. SNP1(G/A): The PCR amplification, SSCP type and sequencing of PCR products by primer P2. The letters inside the figures indicate genotypes.

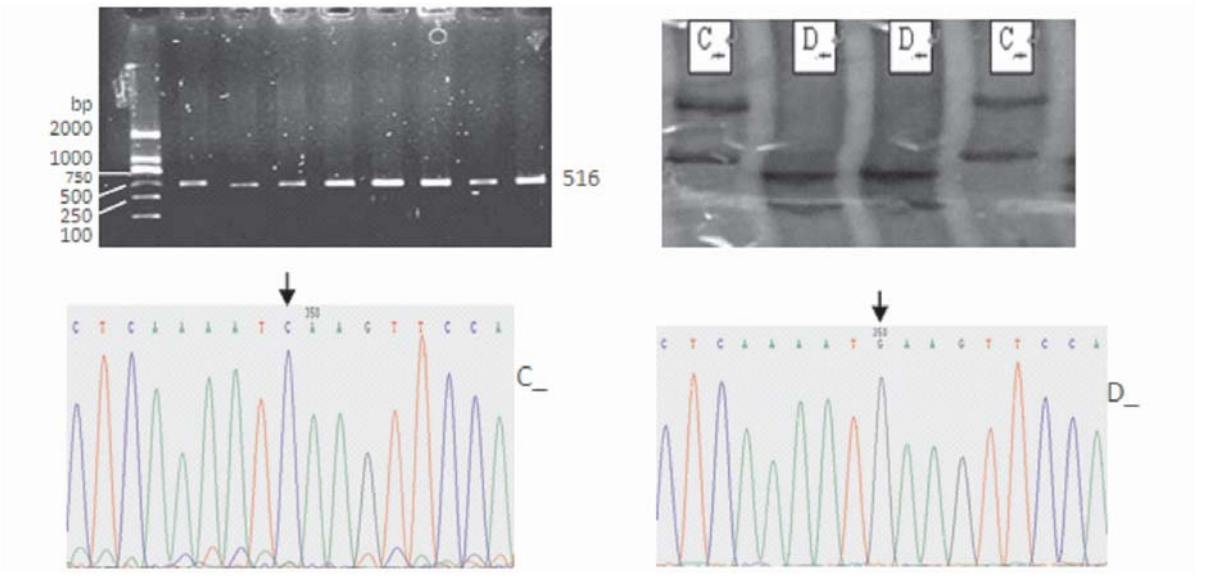

Fig. 2. SNP2(C/G): The PCR amplification, SSCP type and sequencing of PCR products by primer P3. The letters inside the figures indicate genotypes.

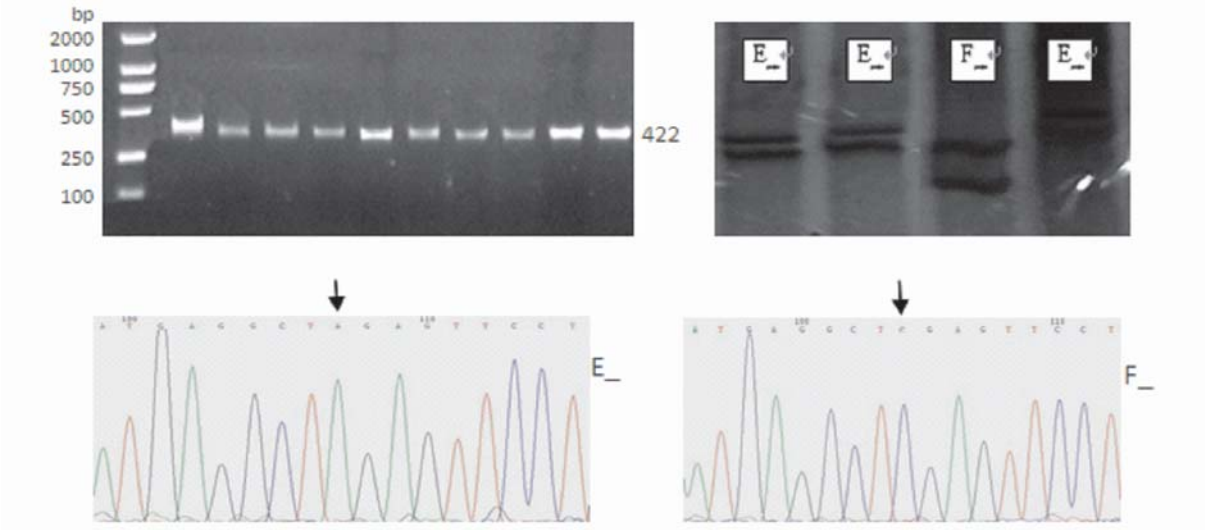

Fig. 3. SNP2(C/G): The PCR amplification, SSCP type and sequencing of PCR products by primer P5. The letters inside the figures indicate genotypes.

Table 2

Frequencies of *HMGCR* gene and genotype

Polymorphism	Mutation sites	Nucleotide mutation	Allelic	Genotype	Frequency	PIC
SNP1	789(intron1)	G A	A B	A_ B_	0.3925 0.6075	0.3551
SNP2	937(intron1)	C G	C D	C_ D_	0.7725 0.2275	0.3204
SNP3	2316(intron4)	A C	E F	E_ F_	0.5075 0.4925	0.3749

Table 3

Genetic analysis of single SNPs on reproduction traits

Location	Genotype	Numbers	Number of eggs	Hatching rate of fertilized eggs (%)	Plasma $_{\text{VLDL content}}$ (mg/ml)
SNP1	A_ B_	157 243	90.19±9.86B 102.14±10.07A	95.07±8.27 95.15±7.03	555.48±133.26A 341.41±141.48B
SNP2	C_ D_	309 91	97.74±11.53 96.53±11.67	94.76±8.09 96.22±5.62	273.45±120.41B 502.44±109.40A
SNP3	E_ F_	203 197	99.65±12.48 A 94.70±9.63 B	94.88±7.74 95.63±7.21	396.44±120.54B 470.01±172.22A

Note: Comparisons were carried out in two genotypes of the same SNP. Different capital letters indicate significant differences ($P<0.01$).

Table 4

Genetic effects analysis of different haplotypes on reproduction traits

Haplotype	Numbers	NE	HRF (%)	C_{VLDL} (mg/ml)
ACE	48	84.90±7.75C	94.83±9.31	184.40±56.79D
ACF	47	93.19±7.34B	–	330.98±168.09C
ADE	30	92.12±10.54 B	–	502.12±118.31A
ADF	32	95.33±11.74 B	–	467.30±93.21A
BCE	115	106.11±8.57A	94.00±7.25	314.30±177.36C
BCF	99	95.27±8.79 B	97.08±6.96	395.75±159.25B

Note: Comparisons were carried among pairs of haplotypes. Different capital letters indicate significant differences ($P<0.01$).

Discussion

As an important candidate gene that affects cholesterol metabolism, polymorphisms of the *HMGCR* gene and their associations have attracted much attention in mammals. However, few mutations of the *HMGCR* gene have been described in chicken. In this study, the chicken *HMGCR* gene, including 6 linear DNA sequences, was cloned using six primer pairs. The PCR products of P2, P3, and P5 had SNPs. The frequency distributions of the *HMGCR* genotypes were different among three different fragments that were obtained by six primer pairs. P1 amplified a high frequency of genotype B_ and allele B. The locus showed intermediate polymorphism, and the chi-

square test indicated that the locus in the population was in Hardy-Weinberg disequilibrium (P>0.01). The Hardy-Weinberg disequilibrium might not be attributed to mutations that lead to the genetic dominance of resistance to mastitis but need long-term selection. Chickens of genotype B_ had a higher number of eggs (102.14 ± 10.07) than those of genotype A_ (90.19 ± 9.86). Chickens of genotype E_ had a higher number of eggs (99.65 ± 12.48) than those of genotype F_ (94.70 ± 9.63). The relatively high frequency of alleles B and E further indicated that the B and E alleles of the *HMGCR* gene were possibly associated with greater egg production. One *HMGCR* SNP that regulates exon 13 skipping is in tight linkage disequilibrium with another intronic *HMGCR* SNP that is associated with low plasma low-density lipoprotein cholesterol (LDL-C) levels by a genome--wide association study in multiple independent populations (BURKHARDT *et al*. 2008; KATHIRESAN *et al*. 2008). A panel including this SNP and 11 others that were associated with levels of either LDL-C or high-density lipoprotein cholesterol was reported to be predictive of cardiovascular disease risk independently of lipid levels (KATHIRESAN *et al*. 2008). In this study, the differences between these haplotypes of the *HMGCR* gene were reflected in the egg production and plasma VLDL concentration. The BCE haplotype had the highest egg production, and the ACE haplotype had the lowest egg production. The average egg production of the BCE haplotype was higher than that of genotypes B_ and E_. These results indicated that haplotype selection to increase egg production is feasible and that it is better than single SNP selection. The haplotypes with higher and lower plasma VLDL concentrations showed lower egg production than other haplotypes, which indicated that a moderate plasma VLDL concentration favors the formation and development of oocytes and eggs.

HMGCR was considered to be a logical candidate gene for cholesterol metabolism because HMGCR is the rate-limiting enzyme in cholesterol synthesis. One *HMGCR* haplotype and its SNPs were associated with increased insulin resistance (XU *et al*. 2010). Insulin regulates energy production, fat metabolism, protein synthesis, glucose utilization, and cholesterol utilization (COLGAN 1993). If, as a result of a low insulin level, glucose cannot be utilized by body cells, it is converted into fat and stored in fat cells. Increased insulin concentrations in laying hens that were induced by feeding dietary chromium and zinc may be related to improved egg production (SAHIN *et al*. 2002). The chickens in this study were past their laying peak, and the pancreas insulin secretion increased and consequently resulted in more cholesterol synthesis to synthesize protein for egg production. Our results indicate that HMGCR may be related to egg production, although the molecular mechanism of how HMGCR variants influence egg production is not currently known. HMGCR might be a factor that modulates the production or secretion of hepatic VLDL, given that cholesterol is important for oocyte and embryo development and that VLDL is the main carrier of cholesterol in laying hens, which affects the content of cholesterol in oocytes and egg yolk. Considering the role of HMGCR in cholesterol synthesis, the activity of HMGCR may be increased by a positive feedback mechanism when chickens reach the laying peak, resulting in increased production of cholesterol. This hypothesis deserves further study.

In conclusion, three base substitutions (G-789-A, C-937-G, and A-2316-C) in the HMGCR gene were found by SSCP and sequencing. The genotypes of single nucleotide polymorphism 1 (SNP1) and SNP3 were significantly associated with the number of eggs. Of the three SNPs of the HMGCR gene, those genotypes with lower plasma very low-density lipoprotein (VLDL) concentrations had higher egg production. Thus, our genetic results suggest that particular variants of the *HMGCR* gene are associated with chicken egg production and that haplotypes with higher and lower plasma VLDL concentrations showed lower egg production than other haplotypes.

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